Human CD4$^+$ T Lymphocytes with Remarkable Regulatory Functions on Dendritic Cells and Nickel-Specific Th1 Immune Responses

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The contribution of T helper (Th) and T cytotoxic (Tc) type 1 lymphocytes in the expression of allergic contact dermatitis to haptens has been amply documented. Conversely, the existence of T cell-based regulatory mechanisms has been poorly investigated. Here, we examined the properties of a subset of nickel-specific CD4$^+$ T cells displaying the cytokine profile (IL-10^{+++}, IL-5^{+++}, IFN-$\gamma^{+/-}$, IL-4$^{+/-}$) of T regulatory cells 1 (Tr1) and with the potential to down-modulate immune responses to nickel. Tr1 clones were isolated from skin challenged with $NiSO_4$ and peripheral blood of nickel-allergic patients, and from the blood of healthy individuals. Tr1 clones expressed CD25, CD28, CD30, CD26, and the IL-12 receptor β2 chain upon activation, whereas the lymphocyte activation antigen-3 was present on 50% of the clones. Monocytes precultured with Tr1 cells in the presence of nickel, or treated with Tr1-derived supernatant, exhibited a markedly diminished capacity to stimulate nickel-specific Th1 responses. Tr1 supernatants also blocked the differentiation of dendritic cells (DC) from monocytes, as well as DC maturation and IL-12 production induced by lipopolysaccharide. As a consequence, the ability of DC to stimulate nickel-specific Th1 and Tc1 responses was greatly impaired. These inhibitory effects were completely prevented by IL-10, but not IL-5, neutralization. In aggregate, the results indicate that Tr1 cells can potently regulate the expression of Th1-mediated allergic diseases via release of IL-10.

Key words: allergic contact dermatitis/interleukin 10/regulatory T cells. J Invest Dermatol 14:295–302, 2000

Allergic diseases are the consequence of exaggerated immune responses against non-self and harmless antigens. Low molecular weight chemicals and drugs, collectively called haptens (Cavani *et al*, 1995; Grabbe and Schwarz, 1998; Griem *et al*, 1998), are frequently responsible for human allergies. In particular, allergic contact dermatitis (ACD) to nickel (Ni) is the most common skin allergic reaction (Diepgen and Conraads, 1997), and it represents a feasible model of T helper 1 (Th1) cell-mediated diseases. ACD has a chronic course with relapses occurring at every contact with the hapten, and is caused by the recruitment into the skin of hapten-specific Th1 and T cytotoxic 1 (Tc1) cells that mediate the tissue damage through the release of pro-inflammatory cytokines and direct cytotoxicity (Albanesi *et al*, 1999; Kehren *et al*, 1999). Type 2 cytokines as well as IL-10 are also represented in ACD skin (Ohmen *et al*, 1995; Werfel *et al*, 1997; our unpublished observation), but their role in the human disease is unknown. In the mouse model, contact hypersensitivity (CH) to haptens is enhanced by IL-12 (Muller *et al*, 1995; Riemann *et al*, 1996) and downregulated by IL-10 (Enk *et al*, 1994; Ferguson *et al*, 1994; Schwarz *et al*, 1994), whereas the effects of IL-4 are still controversial (Berg *et al*, 1995; Asada *et al*, 1997; Traidl *et al*, 1999). Moreover, the expression of murine CH depends on the balance between CD8$^+$ T cells, which exert primarily an effector function, and CD4$^+$ T cells, which may have either effector or regulatory roles (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Xu *et al*, 1996; Bouloc *et al*, 1998; Kehren *et al*, 1999). This last hypothesis is also supported by recent data obtained in humans (Cavani *et al*, 1998; Moulon *et al*, 1998).

In the last few years, it has become evident that specialized CD4$^+$ T cells may perform immunoregulatory functions through the release of suppressive cytokines. For example, TGF-β producing CD4$^+$ T cells, named T helper 3 (Th3), have been shown to suppress both Th1- and Th2-mediated autoimmune diseases (Chen *et al*, 1994; Fukaura *et al*, 1996; Bridoux *et al*, 1997). More recently, a unique subset of IL-10 producing CD4$^+$ T cells has been isolated *in vitro* and named T regulatory cells 1 (Tr1) (Groux *et al*, 1997). Tr1 cells inhibited T cell proliferative responses in an IL-10-dependent manner, and prevented the development of Th1-mediated inflammatory bowel disease in mice. IL-10 is a potent anti-inflammatory cytokine produced by various cell types, including T lymphocytes, monocytes and dendritic cells (DC) (de Waal Malefyt *et al*, 1991; Del Prete *et al*, 1993; De Saint-Vis *et al*, 1998), which downregulates immune responses mainly by inhibiting monocyte and DC presenting capacity (Ding *et al*, 1993; Enk *et al*, 1993; Buelens *et al*, 1997; Morel *et al*, 1997; Steinbrink *et al*, 1997; Allavena *et al*, 1998; O'Farrell *et al*, 1998).

Here we investigated and characterized the functional properties of Ni-specific CD4$^+$ T cells with a Tr1 pattern of cytokine release,

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Abbreviations: ACD, allergic contact dermatitis; Ni, nickel; Sup, supernatant; TCC, T cell clone; Tr1, T regulatory cell 1.

isolated from both peripheral blood and lesional skin of patients allergic to Ni as well as peripheral blood of nonallergic individuals. Ni-specific Tr1 cells were found to inhibit in an IL-10-dependent manner the antigen-presenting cell (APC) function of monocytes and DC, and directly suppress Ni-specific Th1 responses. These results suggest an important role for Tr1 cells in the regulation of Th1-mediated allergies in humans.

MATERIALS AND METHODS

Subjects Four nonatopic patients (24–38-y-old) with ACD to Ni and three healthy, nonatopic volunteers (27–41-y-old) were included in the study. Both patients and control subjects were tested with a patch test series that included $NiSO_4$ 5% in petrolatum (International Contact Dermatitis Research Group Series) applied on the back under occlusion, and evaluated after 48 and 72 h. Patients had a history of eczematous dermatitis after contact with metals and a positive patch test to Ni, whereas nonallergic individuals had never suffered from ACD and had a negative patch test to Ni. Both patients and controls were not taking any medication for at least 15 d before skin testing or blood donation. Skin and blood samples were obtained after informed consent.

Ni-specific T cell lines and clones Skin biopsies (4 mm punch biopsies) were performed under local anesthesia on 48 h positive patch test reactions to $NiSO_4$. After extensive washing in phosphate-buffered saline, biopsies were placed in culture at 37°C with 5% CO_2 in RPMI 1640 complemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential aminoacids, 0.05 mM 2-mercaptoethanol, 100 U per ml penicillin, and 100 μg per ml streptomycin (all from Life Technologies, Chagrin Falls, OH) (complete RPMI) additioned with 5% autologous plasma and 30 U per ml IL-2 (generously provided by Chiron Italia, Milan, Italy). Medium was replaced every third day and, after 12 d, T cells emigrated from tissue samples were collected. Short-term Ni-specific $CD4^+$ and $CD8^+$ T cell lines were also obtained from peripheral blood of both Ni-allergic and healthy individuals as previously described (Cavani *et al*, 1998). Briefly, PBMC were separated by centrifugation over Ficoll-Hypaque (Lymphoprep, Nycomed-Pharma, Oslo, Norway) and left to adhere (6×10^6 cells per ml) in Petri dishes for 2 h at 37°C in complete RPMI with 5% autologous plasma. The nonadherent fraction was depleted of $CD19^+$, $CD56^+$, HLA-DR^+ and $CD8^+$ or $CD4^+$ cells by incubation with immunomagnetic beads coated with specific monoclonal antibodies (MoAb) (Dynabeads M450, Dynal, Oslo, Norway), to obtain > 95% pure $CD4^+$ and $CD8^+$ T cells, respectively. Blood-derived T cell populations were then cultured with irradiated autologous peripheral blood mononuclear cells (PBMC) and 10 μg per ml $NiSO_4$ (Sigma, St. Louis, MO) to enrich for Ni-specific T cells. Both blood- and skin-derived T cell lines were cloned by limiting dilution (0.5 cells per well in 96 well U-bottomed microplates) in complete RPMI plus 10% fetal calf serum (Hyclone, Logan, UT) in the presence of allogeneic feeder cells, 1% phytohemagglutinin (PHA; Life Technologies) and 30 U per ml IL-2. Antigen specificity of T cell lines and T cell clones (TCC) was assessed using irradiated PBMC as APC and 10 μg per ml $NiSO_4$ in complete RPMI supplemented with 5% autologous plasma.

Antibodies The following mouse antihuman MoAb were used: Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (SK3, IgG_1), anti-CD25 (2A3, IgG_1), anti-CD14 (MΦP9, IgG_{2a}), and anti-HLA-DR (L243, IgG_{2a}), pure and FITC-conjugated anti-CD28 (L293, IgG_1), and phycoerythrin (PE)-conjugated anti-CD8 (SK1, IgG_1) MoAb were purchased from Becton Dickinson (San Jose, CA). FITC-conjugated anti-CD1a (HI149, IgG_1), anti-CD86 (2331,IgG_1), anti-CD8 (HIT8a, IgG1), anti-CD30 (BERH8, IgG1) and anti-CD40 (5C3, IgG1) MoAb were from Pharmingen (San Diego, CA). Pure anti-CD3 (UCHT 1, IgG_1), anti-CD83 (Hb15a, IgG_{2a}), FITC-conjugated anti-CD80 (MAB104, IgG_1), PE-conjugated anti-TCR α/β (BMA031, IgG_{2b}) anti-TCR γ/δ (IMMU510, IgG_1), and anti-CD26 (4EL-1C7, IgG1) MoAb were provided by Immunotech (Marseille, France); MoAb (17B4, IgG) against the lymphocyte activation antigen-3 (LAG-3) was a gift from Dr. R. Papoian (Serono, Geneva, Switzerland). Expression of the cutaneous lymphocyte-associated antigen was studied using the rat MoAb HECA-452 (kindly provided by Dr. Louis J. Picker, Laboratory of Experimental Pathology, Department of Pathology, University of Texas South-western Medical Center, Dallas, TX). Control mouse Ig were from Becton Dickinson and control rat IgM from Pharmingen. Secondary FITC- or PE-conjugated goat antimouse IgG were from Dako (Glostrup, Denmark) whereas PE-conjugated antirat IgM was from Pharmingen.

RNA isolation and analysis Total cellular RNA was extracted from resting TCC and TCC activated for 24 h with coated anti-CD3 (1 μg per ml) and soluble anti-CD28 (1 μg per ml), using the acid guanidinium thiocyanate-phenol-chloroform method. For reverse transcriptase polymerase chain reaction (RT-PCR) analysis, 0.5–1 μg of total RNA was reverse transcribed using oligo-dT primers and then subjected to amplification with a GeneAmp RNA PCR kit (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ). The following synthetic oligonucleotides were used: for IL-12R β1 chain, GCTCTAAAGGAG-TATGTTGTCCGC and TTGCACCTGTCTCCATCCTCCAAG (547 bp amplificate); for IL-12R β2 chain, AGATCCAGCAAATAG-CACTTGCGC and AGGTGAGTGGTGAAGAGAACTTG (523 bp amplificate). As an internal control for the amount of RNA used, the β-actin house-keeping gene was employed with primers TGACGG-GGTCACCCACACTGTGCCCATCTA and CTAGAAGCATTTGC-GGTGGACGATGGAGGG (660 bp amplificate). To verify the absence of genomic DNA contamination in the RNA samples in some PCR reactions reverse transcriptase was omitted.

Table I. Cytokine profile of skin- and blood-derived Ni-specific Tr1 cells isolated from allergic and nonallergic donors[a]

Clone	Donor	Origin	–	$NiSO_4$	IL-10	IL-5	IL-4	IFN-γ	TNF-α	TGF-β
RF1.5	Allergic	Skin	1.37 ± 0.21	57.22 ± 2.54	2.9	10	0[b]	0.3	2.3	0.2
RF1.26	Allergic	Skin	1.65 ± 0.20	60.69 ± 2.21	1.2	4	0	0.1	0.9	0.2
RF1.36	Allergic	Skin	0.68 ± 0.52	66.78 ± 0.32	3	12	0.8	0.1	1.5	0.1
AR1.24	Allergic	Skin	0.35 ± 0.02	31.86 ± 2,13	2.3	2.8	0.6	0.1	2.9	0
AR1.51	Allergic	Skin	1.43 ± 0.21	106.69 ± 5.61	3.7	> 12	0.8	0	2.3	0.2
AR2.35	Allergic	Blood	2.86 ± 0.01	71.01 ± 0.40	6.3	0.03	0.6	0	ND	ND
PT1.90	Allergic	Blood	0.17 ± 0.09	6.10 ± 0.16	4.9	1.1	0	0.2	ND	ND
AC2.8	Allergic	Blood	0.40 ± 0.11	8.05 ± 0.37	3.2	0.3	0.4	0	4	0.2
AC1.29	Allergic	Blood	1.34 ± 0.27	79.22 ± 0.94	5.5	3.8	0	0.6	1.2	0.3
DM1.17	Nonallergic	Blood	0.15 ± 0.02	7.99 ± 0.19	2.6	1.5	0.1	0.2	ND	ND
DM1.25	Nonallergic	Blood	0.35 ± 0.03	132.28 ± 4.31	1.8	1.1	0	0	0.5	0.6
DM1.43	Nonallergic	Blood	0.21 ± 0.06	46.49 ± 1.93	2.7	ND	0.3	0.2	0	0
G1.17	Nonallergic	Blood	0.56 ± 0.12	4.56 ± 0.83	3.9	1.2	0.3	0.5	2	0
G2.65	Nonallergic	Blood	1.09 ± 0.26	18.44 ± 1.81	3.2	ND	0.2	0.1	4	0
FN2.10	Nonallergic	Blood	0.41 ± 0.04	180.34 ± 9.23	4.7	2.4	0.6	0.1	1.3	0.4
FN2.11	Nonallergic	Blood	0.27 ± 0.10	65.33 ± 1.22	2.6	5.1	0.4	0.3	0.5	0.3
FN2.21	Nonallergic	Blood	2.54 ± 0.67	159.52 ± 2.13	3.4	3.1	0	0.2	0	0.1
FN2.28	Nonallergic	Blood	0.20 ± 0.03	111.56 ± 2.19	3.9	5.2	0	0.1	1.4	0.3

[a]TCC were used as responder cells (5×10^4 cells per well) in a proliferation assay together with irradiated peripheral blood adherent cells (10^5 cells per well) as APC in the absence or the presence of 10 μg per ml $NiSO_4$. ^{3}[H]Thymidine was added in the last 16 h of a 3 d culture. Results are given as mean cpm $\times 10^3 \pm$ SD of triplicate cultures. For cytokine release assay, TCC were stimulated with coated anti-CD3 and soluble anti-CD28 MoAb (both at 1 μg per ml). Supernatants were collected after 48 h and examined for cytokine content by ELISA. Values are expressed as ng per ml per 10^6 cells.

[b]Below the detection limits of the ELISA kit (pg per ml): IL-4 ≤ 4, IFN-γ ≤ 3, TNF-α ≤ 4, TGF-β ≤ 5.

[c]ND, not determined.

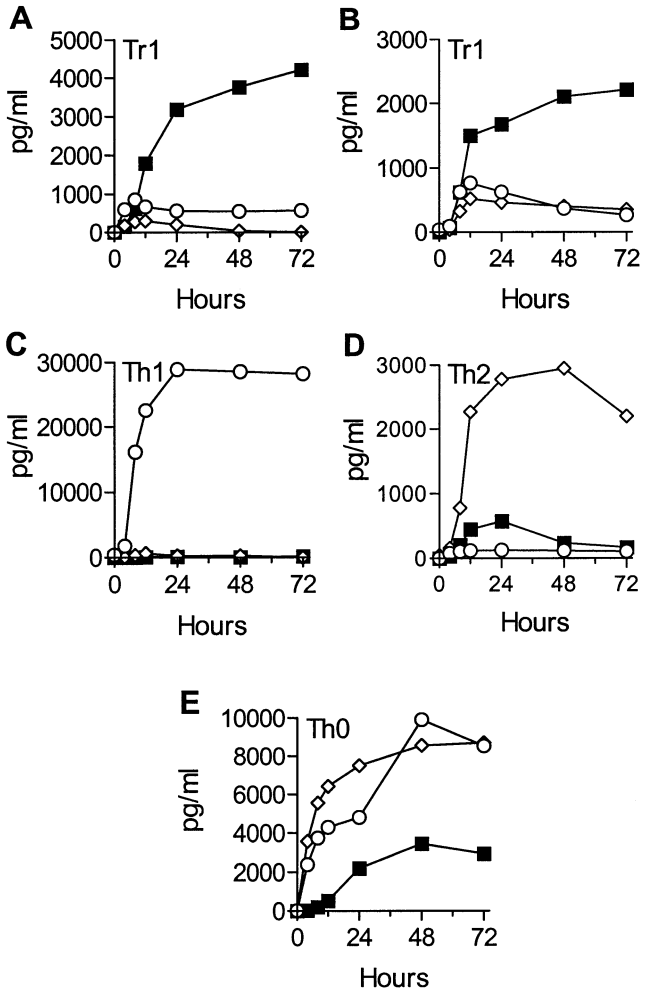

Figure 1. IL-10 is released at early time points by Ni-specific Tr1 cells. T cell clones were stimulated with immobilized anti-CD3 and soluble anti-CD28 MoAb (both at 1 μg per ml). Sups were collected at different time points and examined for IL-10 (■), IL-4 (◇), and IFN-γ (○) levels by ELISA. Two representative Ni-specific Tr1 clones (*A*, AC1.29; *B*, FN2.11) are compared with Ni-specific Th1 (*C*, FN2.4), Th2 (*D*, DM1.46), and Th0 (*E*, AC2.11) clones. A similar kinetic of cytokine release was observed in Ni-specific stimulation assay.

T cell cytokine release Supernatants (Sups) from TCC (10^6 cells per ml) stimulated in 24 well plates with coated anti-CD3 and soluble anti-CD28, or with coated anti-CD3 plus 10^6 irradiated PBMC, were collected after 6–72 h, filtered, and stored at –80°C. In some experiments, TCC were activated with irradiated autologous PBMC in the presence of 10 μg per ml $NiSO_4$. IL-4, IL-5, IL-10, TNF-α, TGF-β, and IFN-γ content was measured by ELISA (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. The pattern of cytokine release of representative Ni-specific Tr1 clones is shown in **Table I**. The Ni-specific Th1 or Th0 clones used as responder T cells in antigen presentation assays or as a source of conditioning sups showed the following cytokine release profile (ng per ml): FN2.4 (IFN-γ, 9.8; IL-4, 0.7; IL-10, 0), FN2.18 (IFN-γ, 3.3; IL-4, 0.5; IL-10, 0.6), AC2.36 (IFN-γ, 3; IL-4, 0; IL-10, 0), RF1.41 (IFN-γ, 7.5; IL-4, 0.1; IL-10, 0.2), and AC2.11 (IFN-γ, 13.6; IL-4, 3; IL-10, 1). The Ni-specific $CD8^+$ Tc1 clone G1.8 released IFN-γ (5.1 ng per ml), but not IL-4 or IL-10.

Monocyte and DC preparations The adherent fraction of PBMC was incubated with 0.2% EDTA for 30 min at 37°C, removed from dishes by scraping, and then depleted of $CD2^+$, $CD19^+$, and $CD56^+$ cells by MoAb-coated immunomagnetic beads. The resulting population (>95% $CD14^+$ cells) is referred to as monocytes. DC were generated by culturing monocytes in complete RPMI supplemented with 10% fetal calf saerum in the presence of 200 ng per ml GM-CSF (Mielogen, Schering-Plough, Milan, Italy) and 200 U per ml IL-4 (Genzyme, Cambridge, MA) at 37°C with 5% CO_2. Medium was replaced after 3 d, and cells were used at day 7 of culture. This procedure gave >97% pure $CD1a^+$ $CD14^-$ DC preparations. To induce maturation, lipopolysaccharide (LPS) from *E. coli* (serotype 055:B5; Sigma) was added at 10 μg per ml for the last 24 h of DC culture. IL-12 (p70) release by DC was determined by ELISA (R&D Systems).

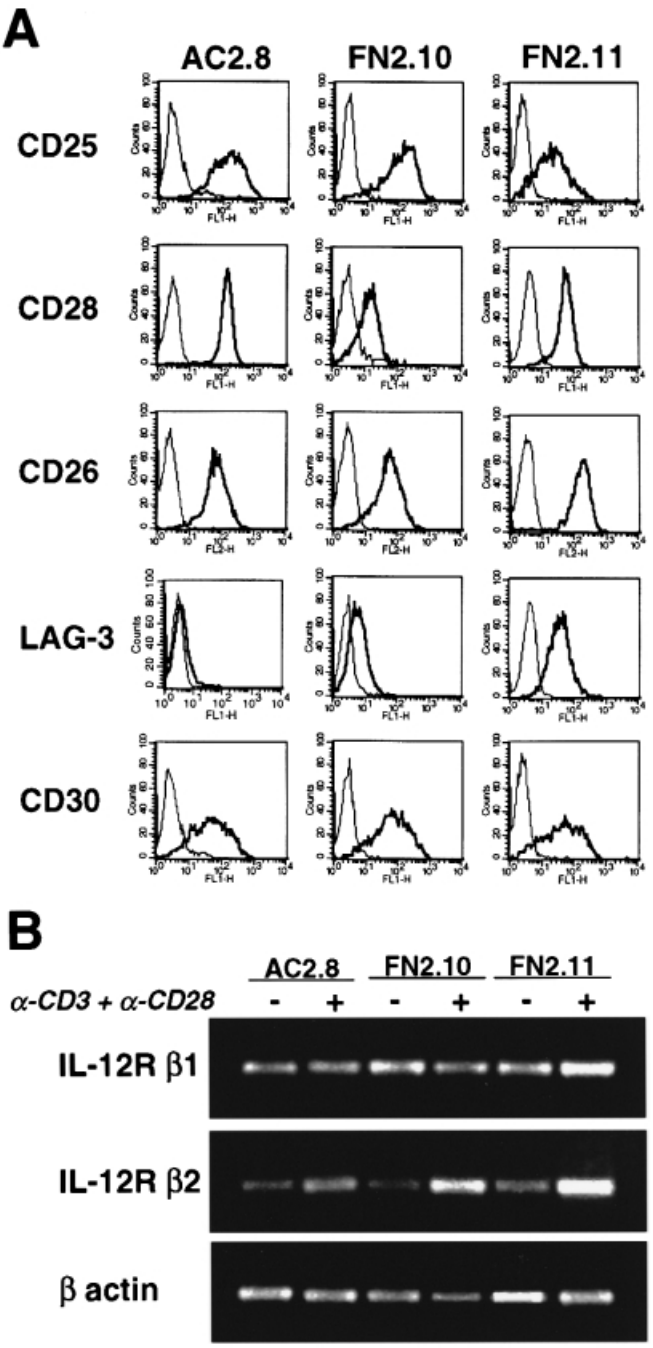

Figure 2. Phenotype of Ni-specific Tr1 cell clones. (*A*) T cell clones were stimulated with irradiated PBMC plus 1% PHA or coated anti-CD3 and soluble anti-CD28, and after 72 h stained with specific MoAb and examined by flow cytometry. Activated Tr1 clones showed a $CD25^+$ $CD28^+$ $CD26^+$ $CD30^+$ phenotype, whereas LAG-3 was detected on 50% of Tr1 clones with a bright or dim expression. Results are representative of staining performed on 20 Tr1 clones. (*B*) RT-PCR analysis revealed the constitutive expression of mRNA for the IL-12R β1 chain in resting T cells, whereas the mRNA for the β2 chain was induced 24 h after activation. Similar results were obtained in two additional Tr1 clones.

Flow cytometry analysis Resting and activated $CD4^+$ TCC or DC were washed with phosphate-buffered saline added with 2% fetal bovine

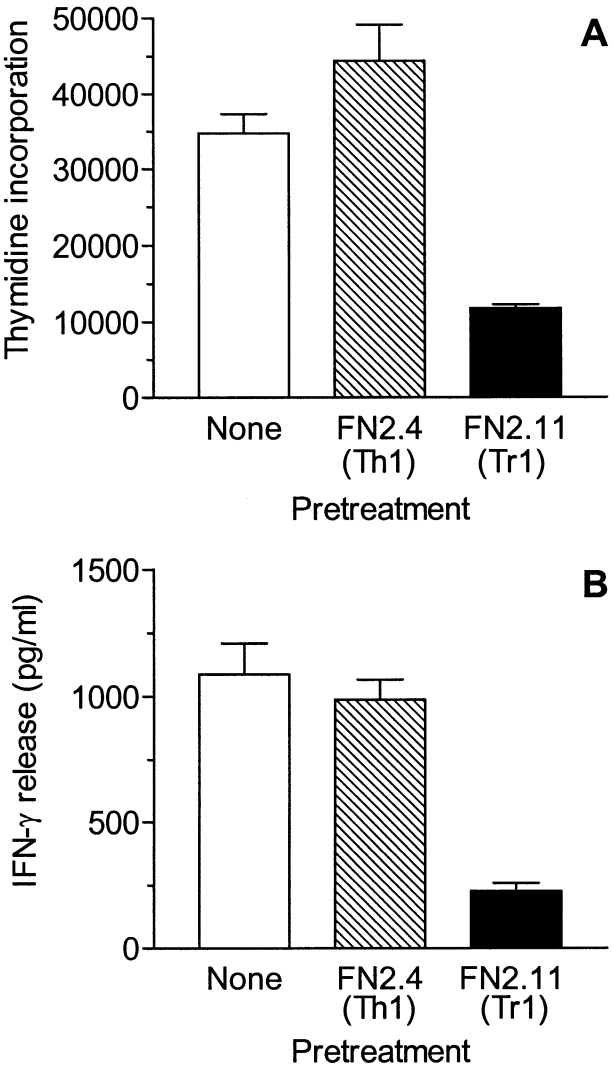

Figure 3. Monocytes preincubated with Tr1 cells and $NiSO_4$ have a decreased capacity to stimulate cell proliferation and IFN-γ release by Ni-specific Th1 clones. Monocytes were cultured with autologous Ni-specific Th1 (clone FN2.4) or Tr1 cells (clone FN2.11) and 10 μg per ml $NiSO_4$ for 24 h. Thereafter, monocytes were collected, depleted of $CD2^+$ cells and finally used to stimulate a Ni-specific Th1 clone (FN1.18). (*A*) Results of proliferation assays are shown as mean cpm ± SD of triplicates. (*B*) Sups were collected at 48 h and IFN-γ content measured by ELISA (pg per ml per 2×10^5 cells). Results are representative of three different experiments.

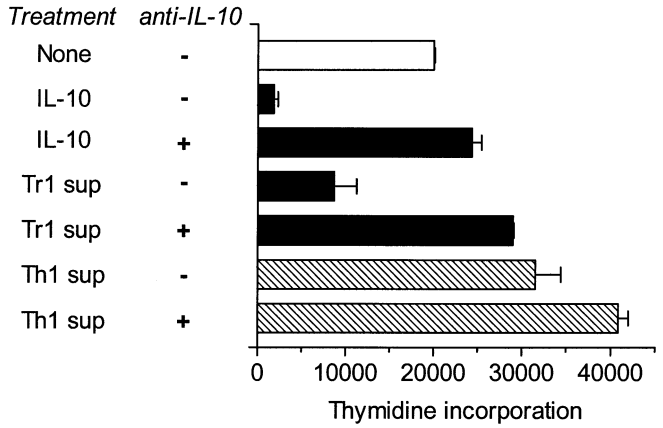

Figure 4. Tr1 cell sup inhibits in an IL-10-dependent manner the capacity of monocytes to activate Ni-specific Th1 cells. Monocytes were incubated overnight with sups from Tr1 (clone FN2.11) or Th1 cells (clone FN2.4) activated with irradiated PBMC and coated anti-CD3 (1 μg per ml), or recombinant IL-10 (10 ng per ml), washed and then used as APC in Ni-specific proliferation assays of a Th1 clone (FN2.18). The monocyte antigen-presenting function was restored when Tr1 sups were preincubated for 30 min at 37°C with an anti-IL-10 MoAb (5 μg per ml). Shown is one representative experiment out of three performed.

serum and 0.01% NaN_3, and then stained with FITC- or PE-conjugated MoAb, or primary MoAb followed by the appropriate secondary FITC- or PE-conjugated antibody. Staining with matched isotype control Ig were included. Cells were analysed with a FACScan equipped with Cell Quest software (Becton Dickinson, Mountain View, CA).

APC conditioning and antigen-presentation assays Monocytes (10^6 cells per well in 24 well plates) were left untreated or incubated with 10^6 Ni-specific Tr1 or Th1 cells in complete RPMI supplemented with 5% autologous plasma at 37°C with 5% CO_2 in the presence of 10 μg per ml $NiSO_4$. After 24 h, cells were collected and T cells removed by negative selection using anti-CD2-coated immunomagnetic beads. Alternatively, monocytes were incubated overnight with culture sups (1:4–1:6 of the final volume) from Tr1 or Th1 cells stimulated with coated anti-CD3 (1 μg per ml) and irradiated PBMC, washed extensively, and finally used to stimulate Ni-specific Th1 cells. Monocytes treated with 10 ng per ml human IL-10 (R&D Systems) for 48 h were also included. For DC conditioning, sups from activated Tr1, Th0, or Th1 cells, or rIL-10 were added at day 0 and 4 of the DC culture. In some experiments, Tr1 sup was added only at day 4 or 12 h before LPS treatment. Anti-human IL-10 or IL-5 MoAb were employed at 5 μg per ml (R&D Systems). Untreated or conditioned monocytes (10^5 cells per well) and DC (2×10^2–10^4 cells per well) were plated in 96 well plates with responder Ni-specific Th1 or Tc1 clones (5×10^4 cells per well) for 60 h or with autologous $CD4^+$ T cells (2×10^5 cells per well) purified from PBMC (bulk $CD4^+$ T cells) for 5 d in the presence of 10 μg per ml $NiSO_4$. In mixed leukocyte reaction (MLR) assays untreated or conditioned DC were incubated 5 d with blood-derived allogeneic $CD4^+$ T cells. Co-cultures were pulsed with 5 μCi per ml ^{3}[H]thymidine (Amersham, Little Chalfont, U.K.) for about 16 h at 37°C, and then harvested onto fiber coated 96 well plates (Packard Instruments, Groningen, The Netherlands). Radioactivity was measured in a beta counter (Packard Instruments). Results are given as mean cpm ± SD of triplicate cultures. In selected experiments, sups from monocyte- or DC-T cell cocultures were collected just before ^{3}[H]thymidine pulsing and assayed for IFN-γ content by ELISA.

RESULTS

Identification and phenotypic characterization of blood- and skin-derived Ni-specific Tr1 cells We have previously shown that Ni-specific $CD4^+$ TCC from peripheral blood of both allergic and nonallergic donors were markedly heterogeneous in terms of cytokine production (Cavani *et al*, 1998). Although most of the specific T cell clones showed a Th1 or Th0 pattern, a subset of blood-derived Ni-specific $CD4^+$ produced high amounts of IL-10 with low or undetectable release of IL-4 and IFN-γ. These cells could not be easily attributed to classical Th1/Th2 subsets. Rather, the cytokine profile of these IL-10^{high} $CD4^+$ TCC was similar to that of the newly recognized suppressor T cell subset, Tr1 (Groux *et al*, 1997). To better understand the role of Ni-specific Tr1 cells in Th1-mediated cutaneous allergies, a panel (n. 50) of IL-10^{high} Ni-specific TCC isolated from both skin lesions and peripheral blood of allergic patients and blood of nonallergic donors were analysed for phenotypic and functional properties. A list of representative clones is shown in **Table I**. Most of the IL-10^{high} Ni-specific TCC examined coreleased high levels of IL-5, whereas IFN-γ and IL-4 were low or absent. TNF-α and TGF-β were variably released, without significant differences compared with Th1 or Th2 clones. Time course experiments showed that IL-10 became the prominent cytokine detected in the sup of Tr1 cells as early as 12 h after activation, with the levels of this cytokine gradually increasing in the following 48–72 h (**Fig 1**). IL-10 was also produced by some Th0 and Th2 clones, but IL-10 release was earlier, higher, and more sustained in Tr1 cells compared with Th0

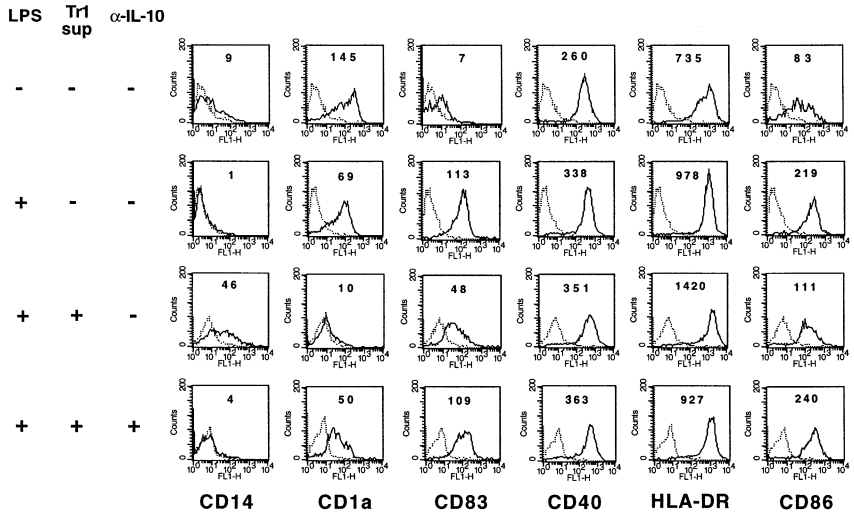

Figure 5. Tr1 sups prevents the development and blocks the maturation of monocyte-derived DC. Monocytes were cultured in the presence of IL-4 and GM-CSF for 7 d to promote DC differentiation. Maturation of DC was induced by adding LPS (10 μg per ml) during the last 24 h of culture. Tr1-derived sup (clone AR1.24; 1:4 of the final volume) was added on day 0 and 4 of culture. Staining was performed as indicated in *Materials and Methods*, and samples were evaluated by flow cytometry. Values represent the mean fluorescence intensity subtracted of the fluorescence of matched-isotype control antibody (dotted lines). Similar results were obtained in five different experiments, and using sups from three distinct Ni-specific Tr1 clones.

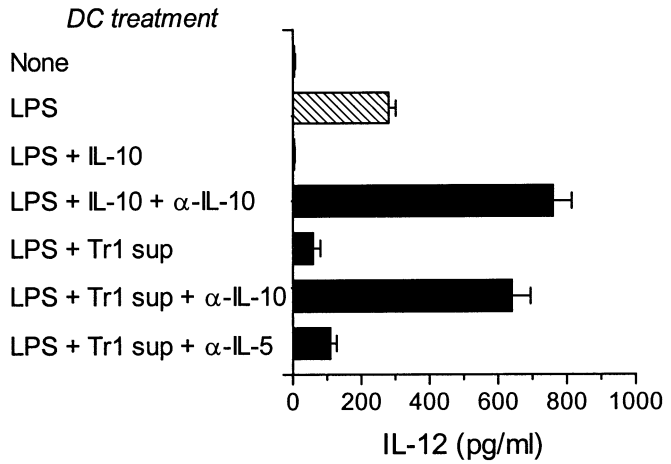

Figure 6. Tr1 sup inhibits the release of IL-12 from mature DC. Monocytes were cultured in the presence of IL-4 and GM-CSF for 7 d to promote DC differentiation. Maturation of DC was induced by adding LPS during the last 24 h of culture. Tr1-derived sup (AR1.24) or recombinant IL-10 (10 ng per ml) were added on day 0 and 4 of culture. Anti-IL-10 or -IL-5 MoAb were used at 5 μg per ml. Data are expressed as pg per ml per 10^6 cells ± SD. Results are representative of two independent experiments.

or Th2 clones. Twenty Tr1 clones were further analysed for expression of T cell markers. Ni-specific Tr1 cells were $CD4^+$, $CD8^-$, TCR-α/β^+, and TCR-γ/δ^-. Upon activation with anti-CD3 and APC, CD25 was uniformly expressed on Tr1 clones. Most of the activated clones (19/20) were $CD28^+$, and expressed CD30 and CD26 (dipeptidyl peptidase IV), whereas LAG-3 was absent on 50%, dim on 20%, and bright on 30% of the Tr1 clones examined (**Fig 2*A***). RT-PCR analysis performed on 3 Tr1 clones revealed also the constitutive expression of the IL-12R β1 chain and, following activation, of the IL-12R β2 subunit, a marker of Th1 differentiation (Rogge *et al*, 1997; Szabo *et al*, 1997) (**Fig 2*B***). The percentage of Ni-specific Tr1 cells was high among TCC isolated from the peripheral blood of nonallergic individuals (25% of 120 $CD4^+$ TCC), and lower but still significant among TCC isolated from peripheral blood (7% of 115 $CD4^+$ TCC) and from the skin lesions (10% of 123 $CD4^+$ TCC) of allergic patients. All skin-derived and about 90% of the blood-derived Ni-specific TCC, including Tr1 clones, were also positive for the skin homing receptor, cutaneous lymphocyte-associated antigen (not shown), thus indicating their capacity to recirculate in the skin (Picker *et al*, 1991; Santamaria Babi *et al*, 1995; Fuhlbrigge *et al*, 1997; Cavani *et al*, 1998).

Tr1 cells inhibit monocyte presenting function through the release of IL-10 IL-10 inhibits immune responses mainly altering the antigen-presenting functions of monocytes and DC (de Waal Malefyt *et al*, 1991; Ding *et al*, 1993; Enk *et al*, 1993; Buelens *et al*, 1997; Morel *et al*, 1997; Steinbrink *et al*, 1997; Allavena *et al*, 1998; O'Farrell *et al*, 1998). To assess whether Tr1 cells could have a regulatory function on APC, monocytes were first cocultured for 24 h with autologous Ni-responsive Tr1 or Th1 TCC in the presence of the relevant antigen. Monocytes were then collected, depleted of $CD2^+$ cells, and finally used for stimulating Ni-specific $CD4^+$ Th1 clones. Results indicated that monocytes that had been preincubated with Tr1 cells, but not with Th1 cells, decreased markedly their capacity to activate Ni-specific Th1 cells (**Fig 3*A***). In parallel to the reduced proliferation, a significant reduction of IFN-γ release was observed (**Fig 3*B***). Inhibition of monocyte function strictly depended on Tr1 activation, as no effects were observed when Tr1 cells were incubated with monocytes in the absence of Ni. To determine whether these effects were dependent on soluble factors released by Tr1 cells, monocytes were cultured overnight with sups obtained from activated Tr1 or Th1 TCC, and, after extensive washing, used for stimulating the proliferation of Ni-specific Th1 TCC. Again, a strong decrease in the capacity of monocytes to activate Ni-specific Th1 clones was observed after treatment with Tr1, but not Th1 sups, and the inhibitory effect was reverted by neutralizing IL-10 with a specific MoAb (**Fig 4**). Monocytes treated with 10 ng per ml of recombinant IL-10 showed marked reduction of their presenting capacity as well. In aggregate, these findings demonstrated that Tr1 sups inhibited the APC function of monocytes through the release of high amounts of IL-10.

Tr1 sup blocks the differentiation of DC from monocytes and DC maturation, and inhibits their presenting capacity DC are critically involved in the induction of CH to haptens (Enk *et al*, 1993; Grabbe *et al*, 1998), and suppression of DC may be crucial to limit the magnitude of the immune response. In the following experiments, we thus examined the effects of Tr1 cell sup on DC differentiation and maturation. DC generated from monocytes with IL-4 plus GM-CSF (Sallusto and Lanzavecchia, 1994), and induced to mature by addition of LPS, were treated with Tr1 sup and then evaluated for the phenotype and APC functions. The addition of Tr1 sup at day 0 and 4 of DC culture strongly inhibited DC differentiation from monocytes, as indicated by the lower CD1a and higher CD14 expression, and blocked the maturation induced by LPS, as showed by the reduced CD83 and CD86 expression. In contrast, we observed an increased expression of HLA-DR, whereas membrane CD40 levels did not change significantly (**Fig 5**). Phenotypic changes induced by Tr1 sup were

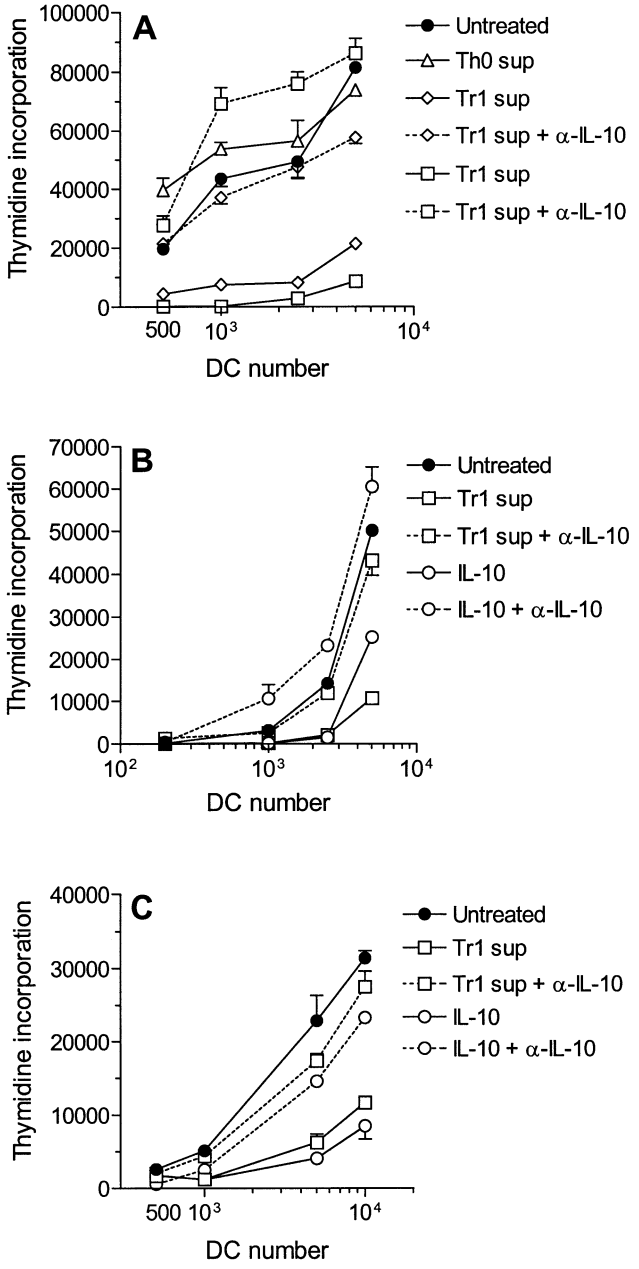

Figure 7. Tr1 sup diminishes DC capability to stimulate both CD4$^+$ and CD8$^+$ T cell responses to Ni. DC were cultured in the presence of sups from either of two Tr1 clones (□, AC2.8; ◇, AC1.29), a Th0 clone (△, AC2.11), recombinant IL-10 (○), or left untreated (●), and then used to stimulate Ni-specific proliferation of peripheral blood bulk CD4$^+$ cells (*A*), a Ni-specific Th1 clone (*B*, FN2.4), or a Ni-specific Tc1 clone (*C*, G1.8). Anti-IL-10 MoAb (dashed lines) was added at 5 μg per ml. Data are expressed as mean cpm ± SD of triplicate cultures. Similar results were obtained in four different experiments.

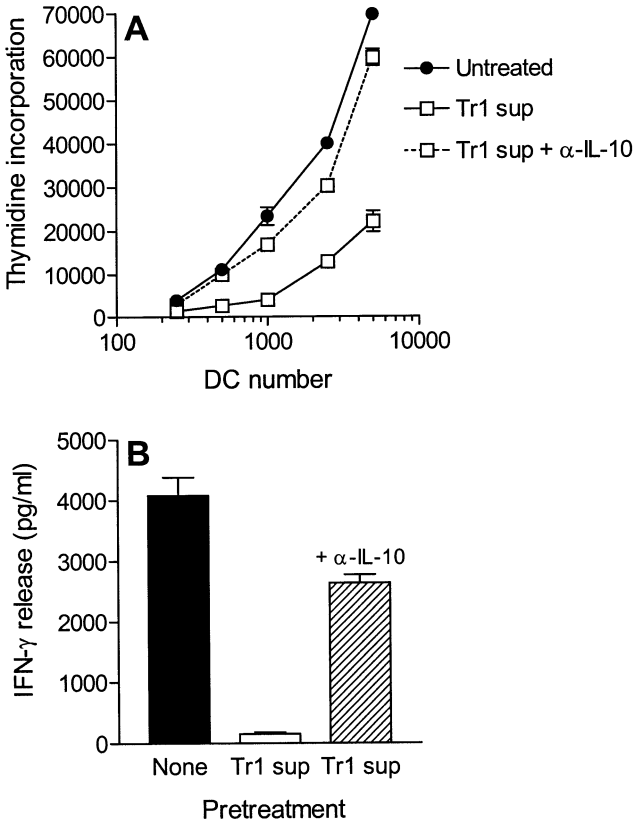

Figure 8. DC treated with Tr1 sup display a reduced capacity to stimulate allogeneic CD4$^+$ T cells. DC, cultured for 7 d in the presence of the sup of the Tr1 clone FN 2.21 (□), Tr1 sup, and anti-IL-10 MoAb (dashed line) or without conditioning T-cell derived sups (●), were incubated 5 d with allogeneic CD4$^+$ T cells (2×10^5 cells per well) in 96 flat bottomed microplates. (*A*) Proliferative responses are expressed as mean cpm ± SD of triplicates; (*B*) Sups were collected at day 4 of DC-T cell coculture. IFN-γ content was measured by ELISA (pg per ml per 2×10^5 cells). Results are representative of three different experiments.

similar to those induced by recombinant IL-10, and could be prevented by addition of an anti-IL-10, but not anti-IL-5 MoAb. Moreover, DC cultured in the presence of Tr1 sup displayed a much lower release of IL-12 upon LPS stimulation (**Fig 6**). Of note, IL-12 release was higher in DC cultures treated with anti-IL-10 MoAb, most likely because anti-IL-10 neutralized also endogenous IL-10 (de Waal Malefyt *et al*, 1991; De Saint-Vis *et al*, 1998). More important, DC exposed to Tr1 sup showed an impaired capacity to stimulate both Ni-specific Th1 and Tc1 clones, as well as peripheral blood bulk CD4$^+$ T cells obtained from an allergic patient (**Fig 7**). Again, inhibitory effects of Tr1 sup on DC functions were completely blocked by addition of an anti-IL-10, but not anti-IL-5 MoAb. Finally, DC exposed to Tr1 sups strongly reduced their capacity to stimulate allogeneic CD4$^+$ T cells, in terms of both cell proliferation (**Fig 8*A***) and IFN-γ release (**Fig 8*B***), indicating that the impaired antigen-presenting function of DC was not antigen-dependent. Tr1 sup had less prominent effects on DC when added exclusively on day 4 of culture, and completely ineffective on DC previously treated with LPS (data not shown), confirming the notion that IL-10 affects immature, but not mature DC (Steinbrink *et al*, 1997; Allavena *et al*, 1998).

DISCUSSION

Tr1 cells were first described as IL-10/IL-5 producing CD4$^+$ T cells obtained *in vitro* after repeated stimulation in the presence of IL-10 (Groux *et al*, 1997). Here, we describe a subset of human Ni-specific CD4$^+$ T cells with a Tr1 cytokine profile (IL-10^{+++}, IL-5^{+++}, IFN-γ$^{+/-}$, IL-4$^{+/-}$) isolated from peripheral blood of healthy donors, and from peripheral blood and lesional skin of patients affected with ACD to Ni. Our findings provide evidence that Tr1 cells do not simply result from the *in vitro* manipulation of CD4$^+$ T cells, but rather they can differentiate *in vivo* and participate in the regulation of an allergic Th1-mediated response.

The origin of Tr1 cells as well as their relationship with other CD4$^+$ T cell subsets is at present unknown. Ni-specific Tr1 cells uniformly expressed the IL-12R β2 chain mRNA (five of five) and most (19 of 20) of the clones expressed CD26, whereas 50% stained with anti-LAG-3 MoAb. These markers have been associated with a Th1-like cytokine profile (Picker *et al*, 1991; Annunziato *et al*,

1996; Szabo *et al*, 1997; Willheim *et al*, 1997; Scala *et al*, 1998). Human Th1 cells can produce variable amounts of IL-10 upon activation (Del Prete *et al*, 1993), and they can inhibit T cell activation and monocyte release of TNF-α when production of IL-10 exceeds that of IFN-γ and IL-2 (Katsikis *et al*, 1995). Our Tr1 cells differ from classical Th1 cells because their production of IFN-γ was very low or undetectable in most cases, and IL-10 was released at early time points after activation. Ni-specific Tr1 clones also expressed (19 of 20) CD30, a T cell marker preferentially, but not exclusively, correlated with Th2 differentiation (Del Prete *et al*, 1995; Nakamura *et al*, 1997). Further experiments are required to determine whether Tr1 represent a divergent maturation step of Th1 or Th2 cells or whether they should be considered a completely different new T cell subset.

Two orders of events suggest that the expression of ACD is a highly regulated phenomenon. First, many individuals present memory Ni-specific $CD4^+$ T cell responses without having developed clinical manifestations of allergy (Cavani *et al*, 1998; Lisby *et al*, 1999). Similarly, it has been shown that the frequency of peripheral blood atopen-specific T cells is comparable in atopic and nonatopic individuals (Cavaillon *et al*, 1988; Sallusto *et al*, 1993), and that $CD4^+$ T cells reactive to myelin basic protein or desmoglein 3 can be isolated from both patients with multiple sclerosis or pemphigus vulgaris, respectively, as well as from normal, healthy individuals (Ota *et al*, 1990; Budinger *et al*, 1998). These findings indicate that the balance between appropriate regulatory and effector mechanisms may be relevant to the development of both allergic and autoimmune diseases. Secondly, despite the long retention of haptens in the skin (Hostynek *et al*, 1993), the inflammatory reaction that follows each hapten application is self-limited, suggesting that local mechanisms may be critically involved in the termination of the immune response. IL-10 producing T cells are good candidates for playing a prominent role in the regulation of the expression of Ni allergy. Ni-specific, Tr1 cells expressing the cutaneous lymphocyte-associated antigen could be easily recruited at skin sites of hapten exposure, and, once activated by the relevant antigen, they can rapidly secrete high amounts of IL-10, which in turn impairs the APC functions of both monocytes and DC, decreasing the activation of effector T cells. Skin DC are the professional APC primarily involved in the initiation and propagation of immune responses to haptens (Enk *et al*, 1994; Bouloc *et al*, 1998; Grabbe *et al*, 1998). Immature DC are very efficient in taking up antigens but display poor T cell stimulatory capacity. Under the influence of inflammatory signals, including cytokines and highly reactive haptens (Banchereau and Steinman, 1998; Kuhn *et al*, 1998), DC undergo maturation and become very efficient in the activation of naïve and memory T cells. DC can differentiate both *in vitro* (Sallusto and Lanzavecchia, 1994) and *in vivo* (Pastore *et al*, 1997) from blood monocytes in the presence of GM-CSF plus IL-4, maintaining the capacity to mature in response to various stimuli. Mature DC strongly increase the expression of costimulatory molecules, augment cytokine release and express the CD83 marker. Monocytes cultured in the presence of IL-4, GM-CSF, and Tr1 sups did not differentiate into DC, retaining a $CD14^{high}$, $CD1a^{neg}$ phenotype. Moreover, DC exposed to Tr1 sups failed to upregulate CD83 and CD86, and to release IL-12 upon treatment with LPS. As a consequence, Tr1-treated monocyte-derived DC stimulated poorly Ni-specific Th1 and Tc1 cells. Both phenotypic and functional changes induced by Tr1 sups on DC were completely prevented by IL-10 neutralization, indicating that the inhibitory activities were strictly IL-10 dependent. The effects of Tr1 sups on DC were prominent when Tr1 sup was added early in the culture, whereas no effects were observed when it was added to already mature DC, as reported previously (Steinbrink *et al*, 1997; Allavena *et al*, 1998). Tr1 sup treatment upregulated MHC class II expression on DC, again in an IL-10 dependent manner. Although some reports indicated that IL-10 decreased MHC class II expression on DC (Steinbrink *et al*, 1997; Allavena *et al*, 1998), our data are consistent with other works showing that IL-10 promotes class II expression in monocyte-derived DC (Morel *et al*, 1997). An additional important mechanism by which IL-10 can depress Th1 responses is by converting DC into tolerogenic APC (Enk *et al*, 1994; Steinbrink *et al*, 1997). Experiments are in progress to see whether Tr1 sups possess a similar activity. In APC-independent T cell activation assays using immobilized anti-CD3 MoAb, Tr1 sups also directly inhibited $CD4^+$, but not $CD8^+$, T cell proliferation, in agreement with results obtained using recombinant IL-10 (de Waal Malefyt *et al*, 1993; Taga *et al*, 1993; Groux *et al*, 1996, 1998) (data not shown); however, this inhibitory effect was only partially blocked by IL-10 neutralization, suggesting that other soluble factors in Tr1 sups can diminish Th1 activation. Although Tr1 cells produce a significant amount of IL-5, this cytokine was not involved in the regulation of DC or monocyte functions, and was not responsible for direct inhibition of Th1 activation in anti-CD3 assays.

In conclusion, Tr1 cells may have a crucial role in limiting excessive tissue reactions to haptens by blocking DC- and monocyte-dependent amplification of hapten-specific Th1 cell responses. The high frequency of Tr1 cells in nonallergic individuals also suggests that these regulatory cells may be important in determining whether silent immune responses or manifest allergic disease to haptens develop. Finally, procedures that selectively amplify this T cell subset may be advantageously used in the immunotherapy of ACD, as suspected to occur during specific immunotherapy of Th2-mediated allergic diseases (Akdis *et al*, 1998).

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